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# Molecular and Genetic Studies of an R Factor System Consisting of Independent Transfer and Drug Resistance Plasmids

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Certain genetic, structural, and biochemical properties of a class 2 R-factor system consisting of the conjugally proficient transfer plasmid I and the naturally occurring non-conjugative tetracycline (Tc) resistance plasmid 219 are reported. I and 219 exist as separate plasmid deoxyribonucleic acid (DNA) species in both *Escherichia coli* and *Salmonella panama*, having molecular weights of $42 \times 10^6$ and $5.8 \times 10^6$, respectively. The buoyant densities of I and 219 are 1.702 and 1.710 g/cm$^3$, respectively, in neutral cesium chloride. Although the Tc resistance plasmid is not transmissible in a normal conjugal mating, it is mobilized in a three-component mating by plasmid I and by certain other conjugative plasmids of the fi$^+$ or fi$^-$ phenotype. Mobilization does not appear to involve intermolecular recombination between plasmids, and no covalent linkage of resistance markers and fertility functions is observed. Transformation of $CaCl_2$-treated *E. coli* by plasmid DNA is shown to be a useful procedure for studying the biological properties of different plasmid molecular species that have been fractionated in vitro, and for selectively inserting non-self-transmissible plasmids into specific bacterial strains. The effects of tetracycline on the rate of protein synthesis carried out by plasmid 219 were studied by using isolated *E. coli* minicells into which this plasmid had segregated. Consistent with the results of earlier investigations showing the inducibility of plasmid-mediated Tc resistance in *E. coli*, the antibiotic was observed to stimulate protein synthesis in minicells carrying the plasmid 219 and totally inhibit $^3$H-leucine incorporation by minicells lacking the Tc resistance marker. Five discrete polypeptide species were synthesized by minicells carrying plasmid 219; exposure of minicells or parent bacteria to Tc resulted in specific and reproducible changes in polypeptide synthesis patterns.

Earlier investigations of the molecular nature of non-chromosomal drug resistance factors (R factors) of the *Enterobacteriaceae* indicate that certain R factors are formed by covalent linkage of plasmids that separately carry resistance or transfer functions (12, 34, 38, 42). The R factors comprising this group have been designated "class 1" by Anderson (4) and "plasmid co-integrates" by Clowes (7); although they exist largely as a single replicon in *Escherichia coli*, members of this class are commonly able to dissociate into component transfer (RTF) and resistance determinant (R determinant) plasmids in *Proteus* (11, 12, 22, 28, 34, 38). It has been suggested that class 1 R factors may contain only a single replicator site which can segregate with either the RTF or R-determinant component of the plasmid when these two units dissociate (45). However, the different replication patterns of the two component units of class 1 R factors in *Proteus* (12, 22, 28, 38) suggest that at least two independent replicator sites are present on R factors of this class. Most previous studies of the molecular properties of R factors have been carried out with members of class 1.

Genetic experiments by Anderson and his collaborators (2, 3), Guinee and Williams (21), and Krcmery et al. (29) have indicated the existence of another general class of R factors.

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Class 2 (4) or "aggregate" (7) R factors exist in *E. coli* as two independently replicating plasmids and are typified by the ΔA and ΔS systems of Anderson and Lewis (3). The R-determinant units of class 2 R factors exist as non-self-transmissible plasmids which can be mobilized by naturally occurring transfer units by a still undefined mechanism.

Genetic (4) and molecular (33) experiments suggest that association between the RTF and R-determinant plasmids of class 2 R factors is not covalent; the plasmids are transferred independently, and transmission of the RTF unit alone often occurs at much higher frequency than transfer of resistance. Moreover, the separate component plasmids of class 2 R factors are transduced independently by phage P1kc (4), in contrast to class 1 R factors which are transduced as a single intact plasmid.

Despite their apparently widespread occurrence, relatively little has been published about the structural characteristics of the RTF and R-determinant components of class 2 R factors. In this report, we describe some molecular and genetic properties of an R-factor system consisting of a conjugally proficient transfer unit isolated from *Salmonella panama* and a naturally occurring tetracycline (Tc) resistance plasmid that is mobilizable but is not self-transmissible. In addition, we report the results of certain investigations of genetic expression of Tc resistance specified by the non-conjugative R-determinant plasmid of this class 2 R-factor system.

## MATERIALS AND METHODS

**Bacterial strains and R factors.** Bacterial strains, plasmids, and relevant phenotypes are shown in Table 1.

**Media.** Bacterial mating was carried out in Penassay broth (PA broth; Difco). The minimal medium (M medium) used in these experiments was prepared by supplementation of the basal minimal medium of Freifelder and Freifelder (20) with glucose (0.5%) and Casamino Acids (0.2%). M medium was supplemented with vitamin $B_1$ (10 μg/ml) for growth of *E. coli* P678-54 and for its derivatives. L broth and L soft agar have been described previously (30, 41).

**Mobilization of Tc resistance plasmid.** A modification of the three-component mating procedure of Anderson and Lewis (3) was employed for mobilization of the Tc resistance plasmid 219. Overnight cultures of bacteria containing the plasmid 219 grown in PA broth in the absence of Tc was diluted 1:3 with similarly diluted overnight cultures of (i) an appropriate donor strain carrying a conjugally proficient plasmid and (ii) a nalidixic acid-resistant final recipient strain. After overnight incubation, mating mixtures were exposed for 2 h to Tc (1 μg/ml) and were then spread on MacConkey agar (Difco) plates containing nalidixic acid (Calbiochem; 50 μg-ml) and Tc (Squibb; 20 μg/ml).

**Isolation of radioactively labeled plasmid DNA.** Bacteria carrying an appropriate plasmid were grown to stationary phase in M medium containing deoxyadenosine (250 μg/ml) and 0.05 μCi of [*methyl*-$^3H$]thymidine per ml (16 Ci/μmol; Schwarz). Cells were collected by sedimentation, and the deoxyribonucleic acid (DNA) extracted by a Brij-lysis method (6) was further purified by centrifugation in cesium chloride gradients containing ethidium bromide (Calbiochem; 500 μg/ml final concentration) (36) as described previously (12).

Covalently closed circular (CCC) plasmid DNA was collected by using a syringe and needle to pierce the side of the centrifuge tube immediately below the CCC DNA band that was visible under ultraviolet light. After removal of ethidium bromide by equilibration with isopropanol (15), DNA preparations were

TABLE 1.

| Bacterial species | Strains | Relevant phenotype | Plasmid | Source |
|---|---|---|---|---|
| *Salmonella panama* | SP219 | Phage type A; carries extrachromosomal tetracycline resistance | 219 | P. A. M. Guinee |
| *S. panama* | SP I | Contains transfer unit; phage type B | I | P. A. M. Guinee |
| *S. panama* | SP477 | Contains R factor specifying ampicillin and streptomycin | 477 | P. A. M. Guinee |
| *S. panama* | SP47N | Chromosomal resistance to nalidixic acid | | P. A. M. Guinee |
| *S. panama* | SP47NR | Mutant of SP47N carrying chromosomal resistance to rifampin | | By selection |
| *S. typhimurium* | SL 1066 | Carries colicinogenic factor, Col I-P9 | Col I-P9 | B. A. D. Stocker |
| *Proteus mirabilis* | PM1 (Rts1) | Carries $fi^-$ R factor, Rts1 | Rts1 | R. Rownd |
| *Escherichia coli* | W3110N | $F^-$; chromosomal resistance to nalidixic acid | | P. A. M. Guinee |
| *E. coli* | W3110NR | Mutant of W3110N carrying chromosomal resistance to refampin | | By selection |
| *E. coli* | RS74 | Carries derepressed transfer unit of the R-factor R1drd19 | RTF-1 | R. Silver |
| *E. coli* | EC-O | Carries F*lac* | F*lac* | S. Falkow |
| *E. coli* | P678-54 | Minicell-producing strain | | R. Curtiss |

dialyzed overnight against TES buffer [0.01 M tris-(hydroxymethyl)aminomethane (Tris), pH 8; 0.01 M ethylenediaminetetraacetic acid (EDTA), pH 8; 0.2 M NaCl] at 4 C. $^3$H-labeled plasmid DNA was isolated from *E. coli* minicells by a lysozyme-Sarkosyl lysis procedure, as described previously (13).

**Centrifugation procedures.** Samples (0.1 ml) of $^3$H-labeled DNA containing $3 \times 10^3$ to $6 \times 10^3$ counts/min were layered onto 5.2-ml 5 to 20% linear sucrose gradients containing 1 M NaCl, 10 mM Tris-hydrochloride (pH 8), and 1 mM EDTA (pH 8). After centrifugation at 20 C for 90 min at 30,000 rpm in a Spinco SW50.1 rotor, fractions were collected through a hole pierced in the bottom of the tube, and a sample of each was assayed (12).

In experiments requiring quantitative determination of the relative amount of CCC DNA, Brij lysates were treated with Pronase and pancreatic ribonuclease as described by Silver and Falkow (procedure B) (42). After subsequent centrifugation in cesium chloride-ethidium bromide gradients as described above, the fraction of closed circular DNA was calculated from the measured area under the satellite DNA peak relative to the total DNA. Analytical centrifugation of DNA in CsCl and calculation of buoyant density have been described (12).

**Transformation of E. coli by plasmid DNA.** $CaCl_2$-treated bacteria (32) were transformed to Tc resistance as described previously (9, 10), except that, after incubation of plasmid DNA with cells for 1 min at 42 C, a 0.5-volume of P broth containing 4 $\mu$g of Tc per ml was added and an additional incubation was carried out for 60 min at 37 C. Samples (0.1 ml) of bacteria were then spread on MacConkey agar plates containing 20 $\mu$g of Tc per ml and incubated for 24 to 48 h at 37 C to select antibiotic-resistant clones.

**Studies of plasmid protein synthesis in minicells.** Plasmid-positive and plasmid-negative *E. coli* minicells were purified by differential centrifugation and two cycles of sucrose gradient centrifugation (13, 37). Preparations of purified minicells obtained from 30-ml bacterial cultures were suspended at 4 C in 1 ml of minimal salts medium containing 0.5% glucose, thiamine (2 $\mu$g/ml), leucine (0.1 $\mu$g/ml), and a mixture of other essential amino acids at appropriate concentrations (13). After addition of $^3$H-leucine (5 Ci/mmol; Schwarz) to a final concentration of 30 $\mu$Ci/ml, incubation of minicells was carried out at 37 C for 150 to 170 min. The reaction was stopped by addition of an equal volume of cold 10% trichloroacetic acid, and cells were rapidly frozen and thawed three times to ensure complete lysis. Incorporation of $^3$H-leucine into hot trichloroacetic acid-precipitable counts was assayed as indicated previously (8).

For experiments involving acrylamide gel electrophoresis of $^3$H-labeled and $^{14}$C-labeled plasmid proteins, minicells were suspended in 0.3 ml of 0.01 M sodium phosphate buffer (pH 7.2) and rapidly frozen and thawed three times. A 0.001-ml amount of a 25% solution of sodium dodecyl sulfate (SDS) and 0.05 ml of $\beta$-mercaptoethanol were added, and the mixture was heated for 1 min at 100 C. The resulting lysate was dialyzed overnight at 4 C against phosphate buffer containing 0.1% SDS and 1% $\beta$-mercaptoethanol. Samples (0.1 ml) of dialyzed lysate were fractionated by electrophoresis through 10% polyacrylamide gels (10-cm length), as described by Weber and Osborn (46), in the presence of a bromophenol-blue marker. After electrophoresis, gels were frozen in dry ice and cut into 1-mm slices. Radioactively labeled polypeptides were extracted from gel slices by shaking each slice overnight in 0.1 ml of 0.1% SDS solution and were assayed in a liquid scintillation counter after addition of 9 ml of a Triton X (33.33%)-toluene-phosphor (66.66%) solution.

**Electron microscopy.** DNA was prepared for electron microscopy by CsCl-ethidium bromide centrifugation of a Brij lysate for 64 h at 40,000 rpm at 10 C. CCC DNA was nicked by deoxyribonuclease (DNase) treatment for 5 or 20 min, as described by Bazaral and Helinski (5). Specimens were prepared for electron microscopy (EM) by using modifications of the spreading technique of Kleinschmidt et al. (27), and were examined at 60 kV with a Philips EM-200 electron microscope. Photographs of DNA molecules were projected on a screen, and their contour lengths were traced with a map-measuring device (12). A grating replica was used for calculation of the magnification factor.

## RESULTS

**Genetic characteristics of SP219.** As described by Guinee and Williams (21), *Salmonella panama* strain 219 (SP219), unlike other Tc-resistant *S. panama* strains, is of phage type A and thus does not restrict phage 47. Although this strain is unable to transfer its Tc resistance in a normal conjugal mating, naturally occurring fi$^+$ and fi$^-$ transfer factors (RTF units) present in *S. panama* isolates were shown to be able to mobilize the Tc resistance determinant of SP219 and thus accomplish its transfer to other bacterial strains. The extrachromosomal nature of the Tc resistance determinant was suggested by the observation that appropriate treatment of SP219 with acriflavine "cured" the strain of this resistance (21).

The ability of certain additional conjugally proficient fi$^+$ and fi$^-$ plasmids to mobilize the Tc resistance determinant of *S. panama* SP219 in a three-component mating is shown in Table 2. As seen in part A of this table, transfer of Tc resistance to either *E. coli* strain W3110N or to *S. panama* SP47N could be accomplished by all plasmids tested except for the isolated transfer unit of R-factor R1*drd*19 (i.e., RTF1) and the temperature-sensitive replication plasmid Rts1 (44). No Tc-resistant clones of W3110N or SP47N lacking the transfer unit were observed on examination of 50 separate isolates, in contrast to the segregation seen after mobilization of certain other drug resistance determinants by transfer unit I (21). Moreover, Tc resistance was uniformly transferable from W3110N or

TABLE 2. *Ability of several sex factors to mobilize the tetracycline resistance in SP219*[a]

| Donor bacterial strain | Conjugally proficient plasmid | Intermediate recipient strain | Final recipient strain | | | |
|---|---|---|---|---|---|---|
| | | | W3110N | SP47N | W3110R | SP47R |
| A. | | | | | | |
| SPI | I | SP219 | + | + | | |
| SP477 | 477 | SP219 | + | + | | |
| OR74 | Hfr F | SP219 | + | + | | |
| EC-0 | F-lac | SP219 | + | + | | |
| RS-74 | RTF-1 | SP219 | − | − | | |
| P678-54 (RTF-1) | RTF-1 | SP219 | − | − | | |
| SL 1066 | ColI-P9 | SP219 | + | + | | |
| PM-1 (Rts 1) | Rts-1 | SP219 | − | − | | |
| B. | | | | | | |
| SP219(Rts1) | Rts-1 | | | | − | − |
| W3110N(I+219) | I | | | | + | + |
| SP47N(I+219) | I | | | | + | + |

[a] Three component matings (part A) were carried out as indicated in Materials and Methods, using a modification of the procedure of Anderson and Lewis (3). The conjugal crosses reported in part B were performed as described previously (12), using a rifampin-resistant recipient. (−) indicates a transfer frequency of $<10^{-9}$, which was the limit of detection in these experiments.

SP47N to an $R^-$ rifampin-resistant recipient in a normal mating (Table 2B). In all instances, intraspecies transfers occurred at a much higher frequency than interspecies transfer of Tc resistance (eg., SPI × SP219 × SP47N resulted in a 30-fold higher transfer of Tc resistance to the final recipient than occurred with SPI × SP219 × W3110N).

**EM of I and 219 DNA.** CCC DNA samples isolated by cesium chloride-ethidium bromide centrifugation of Brij lysates of *S. panama* carrying either I or 219, and of lysates of *E. coli* W3110N carrying both plasmids, were treated with low concentrations of pancreatic DNase, as indicated in Materials and Methods, and were examined by EM. The results of contour length measurements of molecules seen in each preparation are shown in Fig. 1. As seen in this figure, plasmid DNA isolated from SPI showed clusters of molecules having contour lengths approximately 13 μm and 18 to 19.5 μm, in addition to a few molecules having other contour lengths. The majority of DNA molecules isolated from SP219 had contour lengths from 2.8 to 3.0 μm, which corresponds to a molecular weight of about $5.8 \times 10^6$. Plasmid DNA isolated from *E. coli* W3110N carrying both plasmids had a bimodal contour length distribution with approximately equal numbers of molecules at 3 μm and 21 to 22 μm.

**Molecular nature of RTF unit I and Tc resistance plasmid 219.** Together, the transfer unit I and Tc resistance plasmid 219 comprise a class 2 R factor, as defined by Anderson and Natkin (4) and by Clowes (7). $^3$H-labeled DNA from an isolate of *E. coli* strain W3110N which

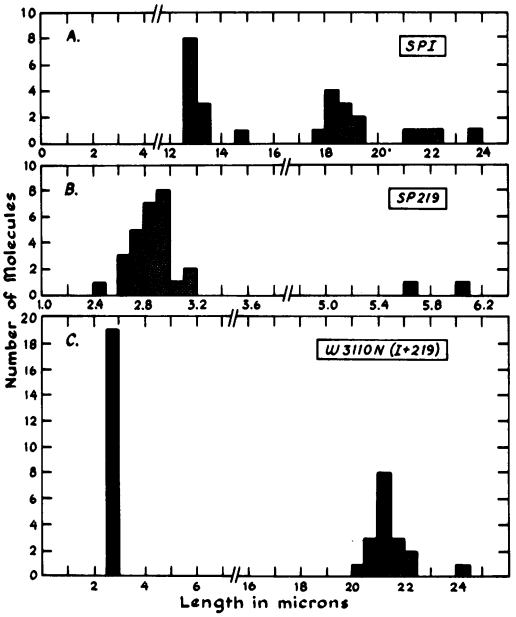


FIG. 1. *Histograms indicating contour length distribution for plasmid DNA species isolated from SPI, SP219, and W3110N (I+219). CCC DNA was isolated from bacteria by Brij lysis and CsCl-ethidium bromide centrifugation, and was nicked with pancreatic deoxyribonuclease (final concentration, 2.5 × $10^{-4}$ μg/ml) prior to examination by electron microscopy.*

had received both I and 219 by the three-component mating procedure was centrifuged in a CsCl-ethidium bromide gradient, and the lower (CCC) DNA band was collected as described in Materials and Methods. After removal of ethidium bromide, this DNA was sedimented in 5 to 20% linear sucrose gradients. Three distinct DNA species having *S* values of 60, 42, and 27 were present in this preparation (Fig. 2). Storage of DNA in TES buffer at 4 C for 1 week resulted in a relative decrease of radioactivity sedimenting in the 60*S* peak and a corresponding increase in size of the 42*S* DNA peak. This observation is consistent with the interpretation that the 60 and 42*S* DNA molecules, respectively, represent covalently closed and nicked circular forms of the same plasmid. The calculated molecular weight (7) for a DNA species having these sedimentation properties is $40 \times 10^6$ to $43 \times 10^6$, which corresponds to the contour length observed for the 19.5- to 21-$\mu$m plasmid molecules isolated from W3110N (I+219) (Fig. 1). The 27*S* CCC peak corresponds to a DNA species having a molecular weight of $5.9 \times 10^6$, suggesting that this peak is the Tc resistance plasmid 219. No peak corresponding to the 13-$\mu$m molecular species isolated from SPI was observed in DNA preparations obtained from W3110N (I+219). Moreover, no 13-$\mu$m molecules were seen on examination of plasmid DNA from W3110N (I+219) by EM. We, therefore, tentatively conclude that the 13-$\mu$m DNA species represents a cryptic plasmid that is coincidentally present in SPI and which was not transferred with I to the W3110N (I+219) isolate that we examined.

A CsCl analytical ultracentrifugation profile of CCC DNA isolated from W3110N (I+219) is shown in Fig. 3. As seen in this figure, DNA species having buoyant densities of 1.702 and 1.710 g/cm$^3$ were present in this preparation. Centrifugation in CsCl of CCC DNA separately isolated from SP219 or SPI, or from the various sucrose gradient peaks shown in Fig. 2, indicated that the $\rho$ = 1.710 g/cm$^3$ DNA species represents the Tc resistance plasmid 219, and that the DNA species at $\rho$ = 1.702 g/cm$^3$ is the transfer unit I.

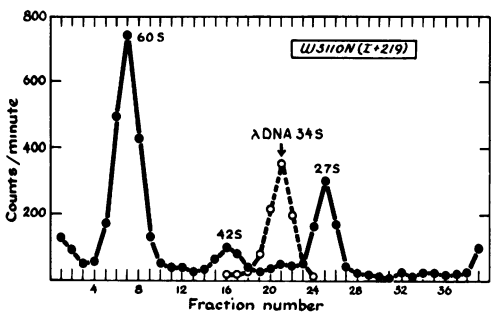


FIG. 2. *Sucrose gradient centrifugation of CCC DNA isolated from E. coli W3110N (I+219)* (●). *The procedures used are indicated in Materials and Methods. No additional DNA species were detected when identical DNA samples were centrifuged for shorter periods of time. Bacteriophage λ DNA which had been heated for 2 min at 70 C was used as a reference marker* (○).

**Transformation of E. coli to Tc resistance.** Fractions comprising the discrete 60, 42, and 27*S* peaks, respectively, from the sucrose gradient centrifugation of circular DNA obtained from W3110N (I+219) (Fig. 2) were pooled and dialyzed against TES buffer, and the DNA from each peak was used separately to transform $CaCl_2$-treated *E. coli* C600 as indicated in Materials and Methods. All three DNA species were able to transform bacteria to Tc resistance; however, the transformation frequency was 100-fold higher for DNA comprising the 27*S* peak (Table 3). Tc resistance was not transferable

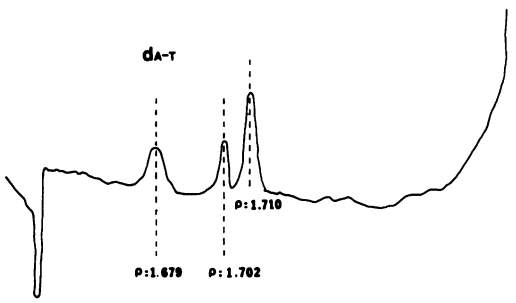


FIG. 3. *Microdensitometer (Gilford) tracing of photograph taken during analytical ultracentrifugation of CCC DNA isolated from W3110N (I + 219) as described in Materials and Methods. Centrifugation in CsCl was carried out for 22 h at 44,000 rpm in the presence of dA-T density marker.*

TABLE 3. *Transformation of E. coli C600 by separated sucrose gradient peaks of DNA isolated from W3110N (I+219)*[a]

| DNA species | Transformants/μg of DNA ($\times 10^{-4}$) |
|---|---|
| I. 60S | 1.5 |
| II. 42S | 40 |
| III. 27S | 420 |

[a] Isolation of DNA and transformation procedure were as indicated in Materials and Methods. The W3110N clone chosen as DNA donor had received Tc resistance by a three-component mating and could transfer this resistance to a recipient strain (Table 2B). DNA peaks are as indicated in the sucrose gradient centrifugation experiment shown in Fig. 2.

from any of five transformants selected from each group; in addition, CCC DNA isolated from colonies selected from cells transformed by each of the three sucrose gradient peaks was examined by centrifugation and was found to contain *only* a 27*S* species. These observations are consistent with our earlier conclusion that the 27*S* ($\rho$ = 1.710 g/cm$^3$) plasmid obtained from *E. coli* (I+219) carries Tc resistance, whereas the larger DNA species that bands at a buoyant density of $\rho$ = 1.702 g/cm$^3$ is the transfer unit I.

These results suggest that transformation for Tc resistance by the DNA contained in the 60 and 42*S* peaks is not the result of recombination of the Tc resistance gene(s) of 219 into I. Rather, it appears that transformation for Tc resistance by the faster-sedimenting peak fractions of the sucrose gradient results from their contamination by small amounts of the 27*S* DNA species. Although our experiments do not rule out the possible transformation of C600 to Tc resistance by a recombinant DNA species containing both I and 219, and subsequent loss of the I component of this putative species, the stability of I and 219 plasmids that are jointly present in a single host after three-component matings makes this appear unlikely.

**Properties of transfer unit I and Tc resistance plasmid 219 DNA in E. coli minicells.** As shown previously, (14, 24, 31, 37) R-factor DNA can segregate into *E. coli* minicells at the time when these spheres are budded off the parent minicell-producing strain. Since minicells lack bacterial chromosomal DNA (1), they represent a useful experimental tool for isolation of R-factor DNA species by biological, rather than physical, methods of separation.

The Tc resistance plasmid 219 was mobilized by transfer factor I to the *E. coli* minicell-producing strain P678-54, and $^3$H-thymidine-labeled minicells produced by the recipient (i.e., P678-54 [I+219]) were purified as indicated in Materials and Methods. Sucrose gradient centrifugation of DNA extracted from minicells by Sarkosyl lysis (Fig. 4A) revealed three distinct peaks having sedimentation properties similar to those of the CCC DNA species that had been isolated from W3110N (I+219). None of these DNA peaks was detected in minicells obtained from the R$^-$ parent strain P678-54 (Fig. 4B) as reported previously (13, 14). These findings suggest that both the transfer factor I and Tc resistance plasmid 219 segregate into minicells and remain as separate circular DNA species in such cells. No Tc-resistant clones of the minicell-producing strain containing only the plasmid 219 were found.

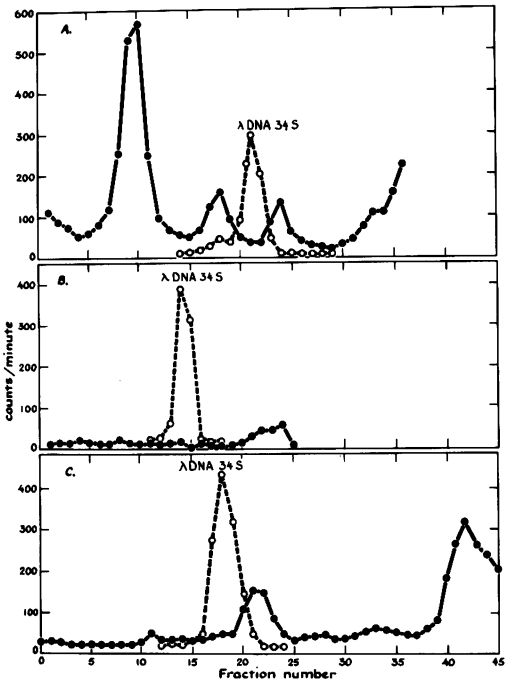


FIG. 4. *Sucrose gradient centrifugation of CCC DNA isolated from E. coli minicells as indicated in Materials and Methods. (A) Minicells derived from bacteria carrying I+219. (B) Minicells derived from P678-54 lacking plasmids. (C) Minicells derived from a clone of P678-54 that has been transformed to Tc resistance as indicated in text. Linear duplex λ DNA served as the reference marker* (O).

**Studies of separate Tc resistance plasmid 219 in E. coli minicells.** Earlier studies have demonstrated that minicells are an effective tool for studying R-factor macromolecular synthesis in the absence of chromosomal DNA, ribonucleic acid (RNA), and protein synthesis (14, 37). Moreover, recent experiments have indicated that protein synthesized by plasmids that have segregated into minicells is biologically functional (A. C. Frazier and R. Curtiss III, Abstr. Annu. Meet. Amer. Soc. Microbiol, p. 170, 1972; 19). It therefore appeared that minicells would be useful for investigation of regulation of the expression of Tc-resistance specified by plasmid 219.

The autonomously replicating 2.8 to 3.0-$\mu$m plasmid 219 contains approximately 7,500 base pairs; a DNA unit of this size can code for a maximum of approximately 2,500 amino acids or about 5 to 10 proteins. The relatively small size of this plasmid and the limited number of genes it can carry made 219 especially suitable for the study of individual plasmid gene products in minicells by using polyacrylamide gel

electrophoresis techniques described previously (14, 37).

Although plasmid 219 was mobilizable to the minicell-producing strain P678-54 by I, the resistance and transfer plasmids both entered minicells (Fig. 4), and attempts to obtain spontaneous segregants of the minicell-producing strain containing only the Tc resistance plasmid were unsuccessful as noted above. However, clones of P678-54 that carry plasmid 219 but lack the transfer unit could be obtained by the transformation procedure indicated in Materials and Methods (9, 10). This was accomplished by using unfractionated CCC DNA isolated from W3110N (I+219), as well as by using the separated 27*S* fraction obtained by sucrose gradient centrifugation of this DNA. Since recombination between I and 219 appears to be rare or nonexistent (Table 3), the low frequency of transformation by plasmid DNA (10) made it unlikely that a single bacterial cell would receive more than one of the molecular species that might be present in a plasmid DNA mixture.

Transformation of *E. coli* P678-54 with a mixture of CCC DNA species obtained from W3110N (I+219) yielded results consistent with this expectation. DNA isolated from minicells derived from three separate transformed clones of P678-54 was examined by sucrose gradient centrifugation; the sedimentation pattern shown by DNA obtained from one of these transformed clones (designated P678-54 [219T1]) is shown in Fig. 4C. As seen in this figure, a single peak having an *S* value of 27 was present in the Tc-resistant transformant; no DNA species characteristic of the faster-sedimenting transfer unit I were seen. Sedimentation profiles of DNA isolated from two other transformed clones, P678-54 (219T2) and P678-54 (219T3), were identical to the pattern shown in Fig. 4C. However, the basal level of Tc resistance conferred by plasmid 219T1 was observed to be approximately two times the level of drug resistance specified by 219T2 or 219T3; moreover, preincubation of cells carrying 219T1 with a small amount of Tc did not affect subsequent expression of resistance to higher levels of the drug to the same extent as it did with 219T2 and 219T3 (J. van Embden and S. N. Cohen, unpublished data). It therefore appeared that expression of Tc resistance may be regulated differently in various transformant clones as compared with each other and as compared with regulation of this resistance in the original *S. panama* species (SP219).

As anticipated, none of the three transformed minicell-producing clones selected was able to transfer Tc resistance to strain W3110N without the assistance of a conjugally proficient plasmid. However, as noted above, the Tc resistance carried by all three plasmids could be mobilized to a third strain by transfer plasmids.

**Protein synthesis in minicells containing plasmid 219T1.** Minicells derived from the plasmid-minus P678-54 parent strain showed little incorporation of $^3$H-leucine into hot trichloroacetic acid-precipitable material, as reported previously (14), whereas significant protein synthesis was evident in minicells containing the plasmid 219T1 (Fig. 5). Moreover, when *E. coli* P678-54 (219T1) was grown in the presence of Tc prior to isolation of minicells, protein synthesis was two to three times higher than was observed when the minicell-producing strain was grown in Tc-free medium. Addition of Tc to purified minicells into which plasmid 219T1 had segregated stimulated the rate of $^3$H-leucine incorporation by these cells (Fig. 5B), whereas protein synthesis in minicells lacking the plasmid was suppressed by Tc.

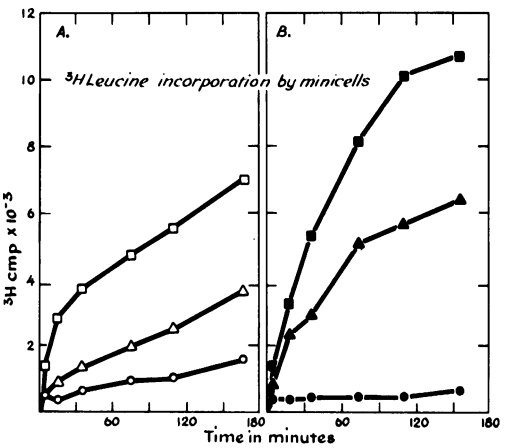


FIG. 5. *Effect of Tc on incorporation of $^3$H-leucine by E. coli minicells. Minicells were isolated and incubated at 37 C in the presence of $^3$H-leucine as described in Materials and Methods. Reactions were stopped by addition of an equal volume of cold 10% trichloroacetic acid, and, after three cycles of rapid freezing and thawing in an acetone-dry ice bath, mixtures were heated for 7 min at 90 C and treated as described previously (8). (A) E. coli P678-54 (219T1) was grown in the presence of tetracycline (12.5 μg/ml) (□) or in the absence of tetracycline (△) prior to isolation of minicells; no tetracycline was present during the minicell incubation; (○) indicates $^3$H-leucine incorporation by minicells derived from the plasmid-minus strain. (B) The minicell-producing strain was grown in the absence of Tc; (■) addition of tetracycline (5 μg/ml) to purified minicells derived from P678-54 (219T1); (▲) no tetracycline; (●) no plasmid, tetracycline added.*

These results are consistent with earlier observations in wild-type *E. coli*, indicating that Tc resistance is inducible (16, 17, 18, 23).

The pattern of $^{3}H$-uridine incorporation into plasmid-positive and plasmid-negative minicells was essentially identical to the pattern shown in Fig. 5 for protein synthesis. In this case also, incubation of the minicell-producing strain with Tc prior to isolation of minicells or incubation of purified minicells with the drug during incorporation of radioactive label resulted in a two- to threefold stimulation of macromolecular synthesis.

Figure 6 shows polyacrylamide gel electrophoresis profiles of lysates of purified minicells which have been incubated for 3 h with radioactive leucine. As seen in this figure, double-label experiments employing $^{3}H$- and $^{14}C$-labeled leucine demonstrate a relative decrease in radioactivity from peak I and a corresponding increase in radioactivity in peaks II, III, and IV as a consequence of Tc exposure. The relative rate of synthesis of polypeptide peak V appears to be unchanged by the drug.

Since the addition of Tc to cultures of the minicell-producing strain carrying the Tc resistance plasmid resulted in a prominent increase in protein synthesis of subsequent isolated minicells, the effects of this antibiotic on replication of the plasmid were investigated. Growth of the P678-54 (219T1) in medium containing Tc led to a 64% increase in the $^{3}H$-labeled plasmid DNA content of minicells derived from this strain (J. van Embden and S. N. Cohen, unpublished data), suggesting that the observed stimulation of RNA and protein synthesis by Tc administration may be secondary to an increase in the number of plasmid copies per bacterial cell. However, attempts to demonstrate an influence of Tc on the replication of plasmid 219T1 in purified minicells were unsuccessful.

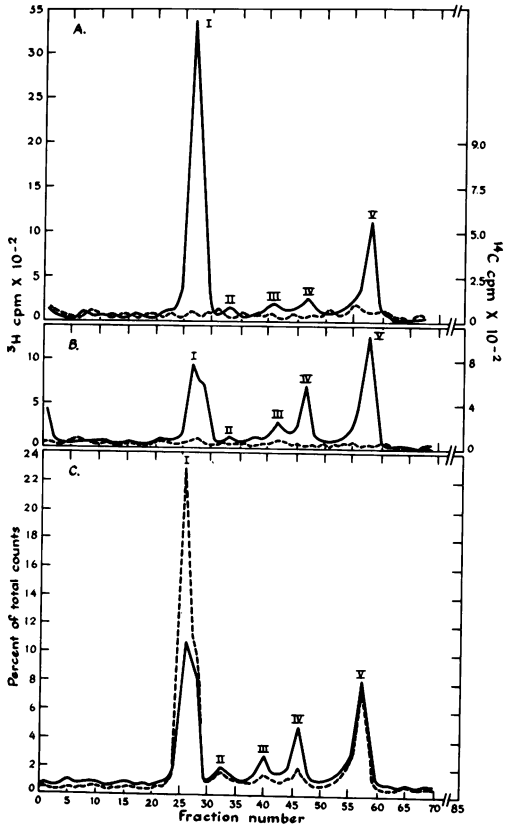


FIG. 6. *Effect of Tc on the synthesis of polypeptide species by E. coli minicells carrying plasmid 219T1. $^{3}H$- and $^{14}C$-labeled polypeptides were mixed in appropriate ratios prior to the heating step and were subsequently treated and subjected to electrophoresis as described in Materials and Methods. Hot trichloroacetic acid-precipitable combined radioactivity (5,000 to 20,000 counts/min) was layered onto each polyacrylamide gel. (A) Tetracycline was absent both during growth of the minicell-producing strain and during incubation of isolated minicells with radioactive leucine; (—) P678-54 (219T1), $^{3}H$-leucine; (- - -) P678-54, $^{14}C$-leucine. (B) Tc absent during growth of minicell-producing strains; Tc present (12.5 μg/ml) during labeling of minicell proteins; (—) P678-54 (219T1); (- - -) P678-54. (C) P678-54 (219T1); (—) Tc present during growth of minicell-producing strain; (- - -) Tc absent. The percentage of total radioactivity in each fraction was calculated separately for $^{3}H$ and $^{14}C$ by dividing the radioactivity of each fraction by the sum of the radioactivity of fractions of the gel.*

## DISCUSSION

The genetic and structural properties of the class 2 R factor examined in our present studies appear to be similar to those reported by Anderson and his collaborators (2, 3, 4) and by Milliken and Clowes (32) for the ΔA and ΔS R-factor systems. In the case of ΔA and ΔS, genetic data have suggested that the drug resistance markers are not covalently linked to Δ after transfer into *Salmonella* or *E. coli*, and recently published structural data (33) support the view that component plasmids of these class 2 R factors exist largely as separate replicons. In the present studies, use of transformation for investigation of the biological properties of separable plasmid DNA species has provided an additional and highly sensitive method for detecting possible recombinant molecules; our failure to observe such species supports the view

that mobilization of resistance determinant plasmids of class 2 R factors does not involve intermolecular recombination. This conclusion is additionally supported by evidence that 219 and other non-self-transmissible plasmids can be mobilized from recombination-deficient bacteria (R. Warkus and S. N. Cohen, unpublished data).

Little is known at present about the mechanism of mobilization of drug resistance determinants by transfer factors. The simplest model suggests that conjugally proficient plasmids accomplish mobilization by their production of pili through which the transfer and R-determinant plasmids can be transferred independently. However, the inadequacy of this explanation and the existence of a more specific mechanism regulating mobilization of plasmids is indicated by our observation that certain of the conjugally proficient plasmids we tested were unable to mobilize plasmid 219, although *all* of the transfer plasmids promoted production of pili and were themselves transmissible. It is of interest that transfer plasmids of both $fi^+$ and $fi^-$ phenotype could mobilize plasmid 219. However, RTF1, which is able to transfer the antibiotic resistance genes of its parent R factor, R1*drd*19, could not transmit the Tc resistance of plasmid 219.

These experiments demonstrate the general usefulness of the plasmid transformation procedure for specifically investigating the biological and genetic properties of discrete molecular species of plasmid DNA that are separable in vitro by sucrose gradient centrifugation or by other fractionation procedures. In addition, transformation has proved to be an effective means of inserting a non-self-transmissible plasmid into *E. coli* minicells, free from a transfer unit. However, it is worthwhile noting that differing biological properties have been observed among several different clones of the minicell-producing strain that have been transformed with a single preparation of plasmid 219 DNA; no explanation for these differences is presently available.

The bacteriostatic antibiotic Tc is a specific inhibitor of protein synthesis in vivo as well as in vitro (18, 25, 35). Studies carried out in several laboratories have shown that, unlike other kinds of bacterial antibiotic resistance, Tc resistance is not the result of modification of protein-synthetic machinery of the bacterial cell or of enzymatic destruction of the antibiotic. Instead, plasmid-borne resistance to Tc is apparently caused by an R-factor-induced decrease in permeability of the bacterial cell to the drug, and a consequently reduced intracellular concentration of the antibiotic. This mechanism has been shown to be involved in R-factor-mediated Tc resistance in *E. coli* (18, 24) as well as in Tc resistance in *Staphylococcus aureus* (23, 40, 43). Additional investigations have demonstrated that preincubation of Tc-resistant bacteria in low concentrations of the drug leads to development of resistance to higher levels of Tc (16, 18, 25). Since induction can be shown after only 1 min of exposure of resistant bacteria to subinhibitory concentrations of Tc (16), it cannot be the result of selection of Tc-resistant cells during bacterial growth.

The recent isolation (17) of an R-factor mutant that is partially constitutive for Tc resistance has given support to the view that an inducible system involving repressor-operator structural gene interactions (26) may be involved in resistance to this antibiotic. The observed effects of Tc on the rate of protein synthesis by minicells containing plasmid 219 (Fig. 5) and on the synthesis of specific polypeptides coded for by 219 (Fig. 6) are consistent with the interpretations stated above. Since isolated minicells do not contain appreciable amounts of chromosomal DNA (1), our observation that Tc resistance can be induced in such cells implies that all of the genetic information required for Tc resistance and for its induction is carried by the plasmid—and that new protein synthesis by chromosomal genes is not necessary during the process of induction. Certain of our data suggest that the observed increase in protein synthesis in minicells that have been derived from bacteria exposed to Tc during growth may result from an increased number of plasmid copies per minicell; however, it is not clear whether this effect is a consequence of selection of resistant bacteria during growth (i.e., a larger percent of minicells contain the plasmid), or from an enhanced rate of replication of the plasmid secondary to exposure to Tc. As noted earlier, we were unable to demonstrate an effect of Tc on the rate of replication of plasmid 219 in isolated minicells.

In contrast to the many polypeptides synthesized in minicells carrying larger drug resistance plasmids such as R6 (14), only five discrete peaks could be attributed to plasmid 219. These approximate the number of genes estimated to be coded for by a plasmid of this size. Alteration of the relative size of four of the five peaks after exposure of minicells to Tc suggests that several of the genes carried by 219 may be involved in either expression or regulation of the Tc resistance it specifies.

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